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Bacterial community structures of an alpine apatite deposit

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Abstract

Apatite represents an important source of inorganic P for natural ecosystems and may favour the establishment of microbial communities able to exploit it. We investigated if microbial community composition in a natural alpine apatite deposit (Piatto della Miniera, canton of Ticino, Switzerland) would differ from the surroundings. Soils were sampled from 3 transects across the apatite deposit and divided in apatite (AP) and non-apatite (NAP) samples. The presence of apatite in the AP samples was confirmed by X-ray diffraction (XRD) and X-ray fluorescence (XRF) analyses. Bacterial community structures were assessed by 16S rRNA gene-based terminal restriction fragment length polymorphism (t-RFLP) and clone libraries. Generally, the AP samples were characterised by higher water-extractable P (e.g. up to 3.1 mg P (g soil dry wt)$^{-1}$), and higher concentrations of organic acids than the NAP samples. t-RFLP analysis showed different bacterial community structures in the AP and NAP samples. The 16S rRNA gene clone libraries of the AP samples were dominated by *Acidobacteria*, *Chloroflexi*, *Actinobacteria*, *Rhizobiales* and *Firmicutes* (relative abundance > 10%), while the NAP samples had a higher relative abundance of *Actinobacteria* and *Chloroflexi* (relative abundance > 20%). The number of different phylotypes related to the dominant groups of the AP clone libraries (11–13) was lower than those in the NAP libraries (17–32). Libshuff analysis of the obtained sequences indicated that the communities developing on the AP are a subpopulation of the flanking NAP sites. Our results suggest that under natural conditions, the presence of rock phosphate may not be the main driving force for bacterial community structures.

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1. Introduction

As components of rocks, minerals contain a diversity of elements, such as P, Zn, Mg and Fe, which represent important micro- and macronutrients for terrestrial ecosystems. Such elements may be released from the minerals through processes of physical, chemical and biological weathering. Microorganisms possess a variety of strategies which enable the release of nutritional elements from minerals. For example, solubilisation through redox reactions and chemical transformations, or the synthesis of chelators, may be involved in microbial-mineral interactions (Gadd, 2010).

In natural ecosystems, the weathering ability of certain microorganisms is particularly relevant when it leads to the release of primary nutrients such as phosphorus (P) (Jones and Oburger, 2011; Welch et al., 2002). P is a macronutrient essential for all living organisms. In natural unfertilised systems, such as young or alpine soils, however, P is often limiting (Turner et al., 2007; Wagai et al., 2011). A major P source for natural environments is represented by rock phosphate, such as apatite, [(Ca5(PO4)3(F, Cl, OH)], which is the most common P-bearing mineral. Apatite deposits are found globally, and are of either sedimentary or igneous origin (Cisse andMrabet, 2004). In Europe, large igneous intrusive apatite deposits are located in Scandinavia and Siberia (Notholt, 1979). However, smaller deposits of igneous origin are also found in central Europe.

Generally, the weathering of apatite occurs synergistically through biotic and abiotic processes, and leads to the release of mineral phosphate. Inorganic phosphate cannot be assimilated by plants, but it can be converted to the bioavailable form orthophosphate (HPO_4^{2-}, $H_2PO_4^-$) by some species of phosphate-solubilising fungi and bacteria. The main mechanism underlying microbial phosphate solubilisation is the secretion of organic acids which, by changing the soil pH and acting as chelators, may induce P dissolution from minerals and its release into the pore water of soils (Vassilev et al., 2006; Welch et al., 2002). The phosphate solubilising ability of many free-living and plant-symbiotic bacterial taxa such as *Pseudomonas* (Browne et al., 2009; Selvakumar et al., 2009; Trivedi and Sa, 2008), Rhizobium (Alikhani et al., 2007; Panhwar et al., 2009) and *Burkholderia* (Lin et al., 2006; Song et al., 2008) has beenwell assessed, as such bacteria play an important role in agricultural applications as plant-promoting microorganisms.

Our present knowledge of the relationship between microorganisms and apatite is based on laboratory incubation experiments and screening-plate assays of cultured isolates, which generally show that a range of fungal and bacterial species are able to solubilise rock phosphate (Chen et al., 2006; Omar, 1998). However, culturing provides only a limited overview of total microbial communities present in environmental samples (Torsvik et al., 1996), as only an estimated 1% of soil microorganisms has been cultivated so far. The assessment and comparison of microbial community structures in the environment requires the profiling of communities based on biomarker genes, such as terminal restriction fragment length polymorphism (t-RFLP) profiling of small subunit (SSU) rRNA genes. More specific information on the microbial taxa present at a site can be obtained by combining the t-RFLP profiles with clone libraries in-silico, or through high-throughput sequencing techniques (Lemos et al., 2011). This strategy may provide useful information on the relation between community structures under different environmental conditions such as a different mineralogical context (Boyd et al., 2007; Lazzaro et al., 2009). Currently, little is known on microbial community composition in natural P-limited ecosystems characterised by high inorganic P, such as natural apatite deposits. The presence of large amounts of this limiting nutrient might select for microbial taxa which are able to exploit it (Mauck and Roberts, 2007). These communities may therefore play a determinant role in initiating the development of young ecosystems, by supplying bioavailable P from the P-bearing minerals (Welch et al., 2002).

The aim of this study was the characterisation of the relationships between bacterial communities and apatite in nature. As a study site, we selected the alpine apatite deposit in Val Cadlimo (Piatto della Miniera, Canton of Ticino, Switzerland), which is one of the largest rock phosphate deposits in Switzerland (Zweifel and De Quervain, 1954). Its peculiarity lies in its location, at approximately 2500 m a.s.l. It represents by a pristine oligotrophic environment dominated by mosses and alpine plants. We focused on the bacterial community present at this site, because bacteria are the most efficient phosphate-solubilising microorganisms (Alam et al., 2002).Wecharacterised and compared community composition in samples taken from the apatite and from flanking regions by t-RFLP of the 16S rRNA gene and 16S rRNA gene clone libraries, providing an insight on dominant bacterial taxa present in the samples.

2. Materials and methods

2.1. Soil sampling

The study site is a band-shaped apatite deposit in Val Cadlimo (Piatto della Miniera, Switzerland) (Fig. 1). The apatite is associated with biotite and quartz and has been previously described by Zweifel and De Quervain (1954). The deposit consists in layers of biotite–apatite and gneiss–schist over a superficial width of 6 to 9 m. The apatite content has been evaluated at up to 24% of the total rock volume with a mineral size ranging between 0.2 and 2 mm.

We selected 3 parallel transects (T1, T2 and T3) that crossed the apatite from N to S. For each transect, sampling spots were selected from the region Northern-flanking the apatite (NAPN), from the apatite deposit (AP), and from the Southern-flanking region (NAPS). Sampling was performed at regular intervals of 2.5 to 5 m along the transect T1, T2, and T3 (Fig. 1), for a total of 5, 13 and 15 sampling sites respectively. At each sampling site, we took composite samples derived from 5 pooled subsamples taken within a radius of 0.3 m.

Soil was collected from the first 5 to 10 cm, after removing the upper vegetated layer, and transferred to plastic bags. Subsamples were homogenised, sieved with a 2 mm sieve and stored at +4 °C until further analysis. In addition, at each location, representative rocks were collected and

stored at room temperature for characterisation of the geological background of the site. Vegetation at each sampling spot was recorded.

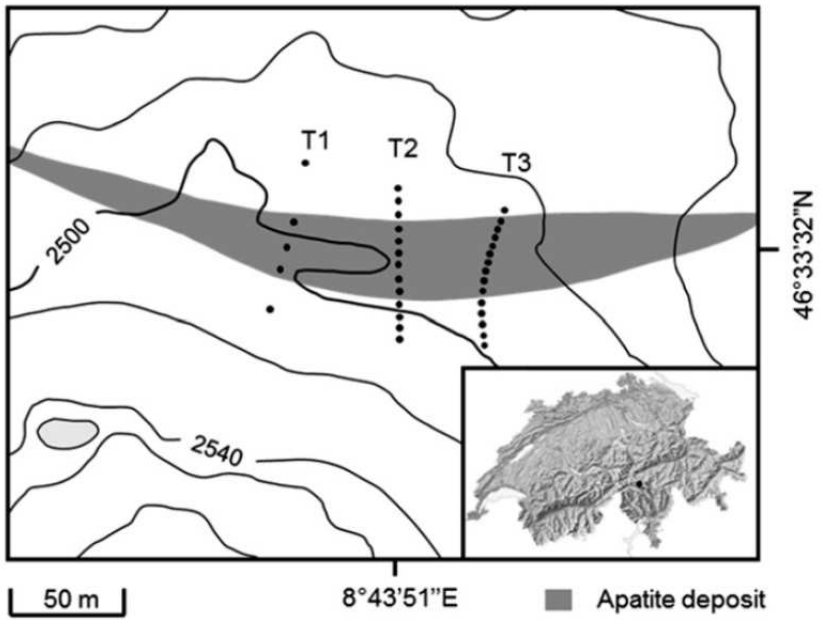

Fig. 1. Location of the apatite deposit (grey) in Piatto della Miniera (Switzerland) with the position of the 3 sampling transects (T1, T2, and T3).

2.2. Geological characterisation

Elemental composition of representative rocks taken from the apatite or in flanking regions was analysed by X-ray fluorescence (XRF). Briefly, approximately 1.5 g milled rock was weighed into graphite crucibles and heated at 1050 °C for 2 h. Following heating, the crucibles were allowed to cool for 10 min and weighed again to estimate loss of ignition (LOI). The samples were then homogenised with 5 times lithium tetraborate (LiB407) before being vitrified on a Claisse M4® fluxer (Claisse, Verrières-le-Buisson, France). Elemental composition of the resulting glass pellets was examined with a wave-length dispersive X-ray fluorescence (WD-XRF) spectrometer (Axios, PANalytical, PANalytical B.V., Almelo, The Netherlands).

The mineralogical composition of the rocks taken from the apatite and from the flanking regions was examined through XRD analysis on a D8 Advance Bruker Powder X-ray diffractometer equipped with a Lynxeye superspeed detector (AD consulting, Prully, CH). Prior to analysis, the milled rocks were dispersed on a glass lens with a few drops of ethanol and then placed on the autosampler. XRD scan was performed at 40 mA at an angle range between 5 and 90°. Mineralogical profiles were compared with the International Center for Diffraction Data (ICDD) PDF-2 database (Release 2005) with the aid of the DEFRAC Plus Evaluation Software.

2.3. Soil chemical properties

For pH measurements, water extractions were prepared by shaking 1 g soil in 10 ml nanopureH2O on an overhead shaker for 1 h. Measurements were made after 10 min of sedimentation with a Calimatic Microprocessor-pH-Meter 761 (Knick GmbH, Corpataux, Switzerland).

The same water extracts were also used for dissolved organic carbon (DOC) determination after filtering through a 0.45 μm filter and acidification with 30 μl of 37% HCl. DOC was measured with a Shimadzu TOC-5000 analyser (Shimadzu SSI, Columbia, MD).

TC and TN contents were measured from 15 mg milled and dried soil on a LECO 932 CHNS Analyser (Leco Corp., ST-Joseph, MI). Dissolved orthophosphate was extracted with water. Organic-acid extractable phosphate was extracted with a solution inspired from the H3A-1 extractant of Haney et al. (2010) that consisted of 1 g l^{-1} sodium citrate, 0.6 g l^{-1} citric acid, 0.4 g l^{-1} maleic acid

and 0.4 g l^{-1} sodium oxalate. Both water- and organic acid-extractions were performed by shaking overnight in an overhead shaker 1 g fresh soil in 10 ml of extractant. Phosphate concentrations in both extractions were determined colorimetrically following the protocol of D'Angelo et al. (2001) using a Synergy HT microplate reader (Bio-Tek Instruments Inc., Winooski, VT). After analyses, the results of all the AP, NAPN and NAPS samples from the different transects were merged.

2.4. Microbial biomass, organic acids and bacterial phosphatase activity

Microbial C biomass was measured from 10 g fresh soil by chloroform fumigation and extraction (CFE) according to the protocol of Vance et al. (1987) using a Shimadzu TOC-5000 analyser (Shimadzu).

Organic acids (see list in Table 3) were extracted from 1 g fresh soil in 5 ml 0.01 M CaCl2 by shaking in an overhead shaker for 1 h, then filtered through a 0.45 μm filter and analysed using an DIONEX IC 320 (Dionex, Sunnyvale, CA).

Phosphatase activity was determined fluorometrically on water extracts according to the protocol of Marx et al. (2001). Briefly, 1 g fresh soil was extracted in 10 ml nanopure H2O in an shaker for 1 h. One hundred microlitres of the soil suspension were pipetted in an opaque microplate and mixed together with 100 μl of 400 μM 4-methylumbelliferone phosphate disodium salt and incubated for 1 h at 30 °C. Dilutions of methylbelliferone in a concentration range 1–100 μM were used as standards. After incubation, ammonium-glycine was added to enhance the fluorescent signal (Hendel and Marxsen, 2005).

2.5. t-RFLP profiling of community structures

DNA extraction from the composite samples was carried out according to the protocol of Noll et al. (2005) from 0.5 g fresh soil. DNA was quantified with the SYBR® Green I method (Leggate et al., 2006) using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments).

PCR amplification of the 16S rRNA geneswas performedwith the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′, FAM-labelled at the 5′ end) and 1401R (5′-CGGTGTGTACAAGACCC-3′) (Weisburg et al., 1991). Approximately 10–20 ng of DNA were added to a PCR master mix containing 1× PCR buffer, 1 mM MgCl2, 0.5 μM of each primer, 0.2 mM of dNTPs, 4% BSA and 0.5 Unit μl^{-1} of GoTaqTM Flexi DNA Taq polymerase (Promega US, Madison, WI). A touchdown PCR program was applied with a first step of denaturation for 3 min at 94 °C, followed by 14 cycles with a denaturing step of 20 s at 94 °C, annealing for 45 s at temperatures between 63 and 56 °C (decreasing 0.5 °C at each cycle), elongation for 1 min at 72 °C. Subsequently, the PCR reaction was characterised by additional 16 cycles as before but with the annealing step at 56 °C for 45 s. The PCR reaction was then terminated with a final step of 4 min at 72 °C. The quality of the PCR products was assessed by gel electrophoresis and SYBR®Safe (Invitrogen Corp, Carlsbad, CA) staining.

Enzymatic restriction was performed by incubating the PCR product with an equal volume of digestion master mix containing 0.1 Unit μl^{-1} of MspI in 1× Tango buffer (Fermentas Inc., Glen Burnie, MA) for 3 h at 37 °C. The digested PCR products were purified using 10% Sephadex™ G50 (Sigma-Aldrich Chemie GmbH, St. Gallen, Switzerland) in Millipore filter plates (Millipore Corp. HQ, Billerica, MA). For t-RFLP, 3 μl purified digestions were first denatured in 10 μl HIDI formamide (Sigma-Aldrich) and 0.1 μl of MapMarker® 100 Rox-labelled Marker (Bioventures Inc., Murfreesboro, TN) at 95 °C for 2 min and immediately chilled on ice. t-RFLP was performed on an ABI 3130XL genetic analyser (Applied Biosystems, Foster City, CA).

2.6. Clone libraries sequencing and analysis

Clone libraries were constructed from PCR products of AP samples deriving from T1, T2 and T3. As references, additional libraries were constructed from T2 north (NAPN) and from T3 south (NAPS).

After PCR as mentioned before, amplicons were purified with Ultra PCR CleanUp Kit (Thermo Fisher Scientific, Wilmington, DE) and quantified. Approximately 25–50 ng μl^{-1} purified PCR products were ligated in the vector CloneJet (Fermentas) and transformed in Oneshot® TOP10 competent cells (Invitrogen) according to the manufacturer's instructions. Transformants were selected for sequencing by a first PCR amplification as described before using the vector-specific primers pJet1.2 forward (5′-CGACTCACTATAGGGAGAGCGGC-3′) and pJet1.2 reverse (5-AAGAACA

TCGATTTTCCATGGCAG-3′), all supplied by Fermentas, and checked through agarose gel electrophoresis.

Prior to sequencing, positives were purified with Sephadex columns and quantified with Nanodrop (ND1000, Thermo Fisher Scientific). The sequencing cycle was performed with approximately 100–150 ng of purified PCR products added to 1 μl Big Dye Terminator V1.1, 1 μl 5× sequencing buffer (ABI) and 1 μl of 1 mM pJET1.2 forward primer. Sequencing included 1 step of 94 °C for 2 min and 60 cycles of 94 °C for 10 s, 50 °C for 5 s and 60 °C for 3 min. Sequencing reactions were purified with the ZR-96 DNA sequencing Clean-up Kit (Zymo Research Crop., Irvine, CA) and ran on an ABI 3130XL genetic analyser (ABI).

Sequences were checked for Chimeras with Pintail V1.1 (Ashelford et al., 2005) and deposited in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/, last accessed on December 15th 2011) database under accession numbers JQ267801– JQ267991. The sequences were between 403 and 444 bp long. Sequence comparison was performed using the NCBI database with a >98% similarity.

The sequences originated from the clone libraries of each transect were merged in an AP and an NAP matrix. DNA distance matrixes were generated for both clone libraries using Multalin (Corpet, 1998) and BioEdit V. 7.0.9.0 (Hall, 1999). Phylotype composition of the AP and NAP. DNA distance matrices was then compared using Libshuff (Singleton et al., 2001).

2.7. Statistical analysis

Analysis of variance of phosphatase activity was performed between AP and NAP samples within each transect (Systat V.12, 2007). For comparing the t-RFLP profiles of the communities located on the apatite (AP) and on the flanking sites (NAP), operational taxonomic units (OTUs) were defined and generated into percentage matrixes using the IBEST (Abdo et al., 2006) program in Perl. The AP and NAP t-RFLP profiles were compared with a principal component analysis (PCA) in SPSS V.19 (IBM® North America, New York, NY) and Adonis analysis of dissimilarities with the vegan package in R (Oksanen et al., 2011).

3. Results

3.1. Vegetation, Rocks and soil properties

The vegetation of the AP region was dominated by plants typical of acidic snowbeds such as *Arenaria biflora, Alchemilla pentafillea, Guaphalium supinum, Salix herbacea and Soldanella* sp. Other common plant species at the sites were ubiquitous alpine plants such as *Leucanthemopsis alpina* and *Phyteuma haemisphaeri*. A high abundance of bryophytes could also be noticed in the AP region.

Elemental analysis of the rocks evidenced differences in major elements between the rocks collected from the AP and the NAP regions. The rocks taken from the AP region appeared richer in several trace elements such as Ba, Sr, Zn, and V. Moreover the P_2O_5 content in the rocks collected from the AP regions (9.38 ± 1.68%) was significantly ($p<0.01$) higher than in those from the NAP regions (0.18 ± 0.02%) (Table 1).

Table 1
Major elemental composition of rocks collected from the AP and NAP regions.

Major elements (%)			Trace elements (ppm)		
Rocks	AP	NAP	Rocks	AP	NAP
SiO_2	37.42 (2.54)[a]	73.43 (1.18)	Rb	359.32 (47.39)	164.70 (33.36)
TiO_2	2.31 (0.33)	0.24 (0.02)	Ba	689.44 (86.11)	1048.74 (337.44)
Al_2O_3	10.87 (1.13)	14.85 (0.54)	Sr	1012.76 (247.98)	186.98 (106.36)
Fe_2O_3	13.36 (1.84)	1.62 (0.36)	Zn	173.98 (15.17)	39.22 (20.89)
MnO	0.12 (0.01)	0.01 (0.05)	V	245.72 (23.99)	15.2 (2.03)
MgO	6.68 (1.32)	0.50 (0.06)	La	124.2 (48.57)	24.6 (6.43)
CaO	13.39 (2.30)	0.59 (0.33)	Ce	382.56 (95.61)	28.2 (16.17)
Na_2O	n.d.	2.65 (0.94)	Nd	129.16 (29.83)	17.26 (5.12)
K_2O	6.13 (0.57)	5.51 (1.16)	Pb	68.44 (2.68)	80.8 (14.35)
P_2O_5	9.38 (1.68)	0.18 (0.02)	U	n.d.	0.28 (0.52)

n.d. = not detected.
[a] Values represent means, while standard deviations are presented in brackets.

Differences could be observed also through XRD analysis of the mineralogical composition of the rocks (see Fig. S1). Rocks collected from the AP regions appeared to be dominantly composed of a mixture of quartz (approximately 30%), phyllosilicates such as biotite, phlogopite and annite (approximately 30%) and fluorapatite (approximately 10%). The rocks taken from the NAP regions were clearly distinguishable, as they were characterised by a dominance of quartz (>49%), followed by muscovite (approximately 23%) and albite (approximately 17%). No traces of apatite could be found in any of the NAP rocks.

We could not observe any significant differences in pH between the AP and the NAPN or NAPS samples. In all soil samples, pH was slightly acidic and ranged between 4.7 ± 0.41 and 4.9 ± 0.38 (Table 2). TC and TN contents were significantly lower in the AP samples than in the flanking sites (Table 2). For example, TC in the NAPN samples was 7.6 ± 4.2% but only 5.0 ± 3.8% in the AP samples, while TN ranged from 0.5 ± 0.2% (NAPN samples) to 0.3 ± 0.2% (AP samples). The C/N ratio was constant in all the NAP and AP samples, ranging from 13.9 ± 4.4 (AP) to 14.7 ± 1.2 (NAPN).

Generally, there were no significant differences in the DOC values between AP and NAP samples, although DOC tended to be higher (0.08 ± 0.05 mg C · (g soil dry wt)$^{-1}$) than in the NAPN (0.05 ± 0.03 mg C · (g soil dry wt)$^{-1}$) and in the NAPS samples (0.06 ± 0.04 mg C · (g soil dry wt)$^{-1}$). Organic acid-extractable P appeared higher than the water-extractable P concentrations (Fig. 2). However, both water- and organic acid-extractable P concentrations showed a significant increase in the AP samples compared to the NAP samples (p b 0.01) (Fig. 2). The highest P concentrations were found in particular in the organic acid-extractable P concentrations from the AP samples (4.2 ± 2.7 μg organic acid-extractable P (g soil dry wt)$^{-1}$).

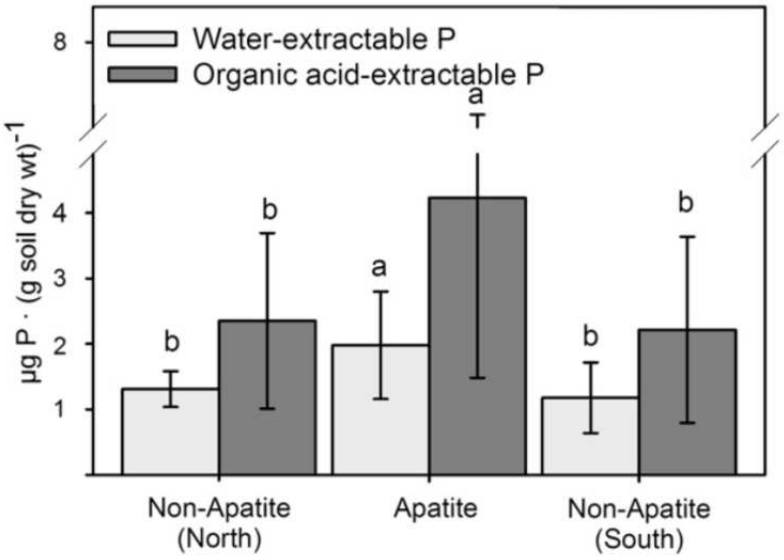

Fig. 2. Concentration of water-extractable P and H3A-extractable P in the samples collected on the apatite deposit (AP) and in northern-flanking (NAPN) and southern-flanking (NAPS) sites. The letters indicate the significant differences ($p < 0.05$ for water-extractable P, $p < 0.01$ for organic acid-extractable P) in P concentrations within the water- and organic acid-extractable P pools.

3.2. Microbial biomass, organic acids and phosphatase activity

Estimations of microbial biomass on the basis of DNA amounts did not follow the same pattern, as the extracted DNA exceeded 200 ng DNA (g soil dry wt)$^{-1}$ in all the samples (Table 2). No significant differences could be observed in either microbial biomass or DNA concentration between AP and NAP samples.

Table 2
Major chemical and biochemical soil properties of the apatite deposit (AP) and of the flanking sites (NAPN and NAPS).

Sample groups	pH	TC	TN	C/N	DOC	Biomass C_{mic}	DNA
		%			mg C · (g soil dry wt)$^{-1}$		ng DNA · (g soil dry wt)$^{-1}$
NAPN	4.90 (0.24)[a]	7.61 (4.21)	0.50 (0.24)	14.74 (1.17)	0.05 (0.03)	37.32 (23.30)	498.92 (64.90)
AP	4.92 (0.38)	5.01 (3.76)	0.33 (0.23)	14.56 (1.43)	0.08 (0.05)	59.30 (37.38)	413.86 (179.09)
NAPS	4.72 (0.41)	6.68 (4.18)	0.47 (0.28)	13.86 (4.45)	0.06 (0.04)	60.37 (28.09)	378.81 (199.05)

[a] Values represent means, while standard deviations are presented in brackets.

Among the organic acids measured, citrate was present in the largest amounts (>244 μmol (g soil dry wt)$^{-1}$), followed by formate (52.84 ± 11.36 to 74.09 ± 89.51 μmol (g soil dry wt)$^{-1}$). Oxalate and pyruvate could not be detected in any of the samples. Generally, we could observe higher concentrations of acetate, formate and lactate in the AP samples in comparison to the NAPN and the NAPS samples. Phosphatase activity (Table 3) was constant and did not seem to be correlated to either, the TC content, the microbial biomass nor to the apatite deposit.

Table 3
Organic acids concentrations and phosphatase activity measured on the apatite deposit (AP) and on the flanking sites (NAPN and NAPS).

Sample groups	Organic acids						Phosphatase activity
	Acetate	Citrate	Formate	Gluconate	Lactate	Propionate	
	nmol (g soil dry wt)$^{-1}$						mM MUB (g soil dry wt · h)$^{-1}$
NAPN	n.d.[a]	374.2 [b] (–)	52.84 (11.36)	9.51 (5.91)	11.57 (1.87)	2.30 (0.43)	2.06 (0.99)
AP	1.13 (4.68)	244.42 (25.58)	74.09 (89.51)	9.10 (5.76)	18.49 (5.97)	2.24 (1.25)	2.20 (0.87)
NAPS	n.d.	n.d.	53.47 (9.45)	5.10 (6.65)	13.00[b] (–)	2.00 (0.68)	2.02 (1.11)

n.d. = not detected (detection limit 2 nmol (g soil fresh wt)$^{-1}$).
[a] Values represent means, while standard deviations are presented in brackets.
[b] Citrate was detected only in one replicate of the NAPN samples. No standard deviation can therefore be provided for this value.

3.3. Bacterial community structure by t-RFLP and sequencing of clone libraries

From the t-RFLP analysis, we obtained and compared 33 t-RFLP profiles with a total number of 70 OTUs. Most of the AP and NAP profiles presented 6 dominant (relative abundance > 5%) t-RFs at 101, 130, 134, 139, 303 and 491 relative migration units (rmu). A few t-RFs in the AP samples (e.g. 72, 126, 306 rmu) were unique or characterised by a high relative abundance. PCA showed only a slight clustering of the AP samples (Fig. 3) along PC1 (32.1%). Adonis analysis (R^2 = 0.05, p = 0.07), however, did not evidence any significant effect of the apatite deposit on the t-RFLP profiles.

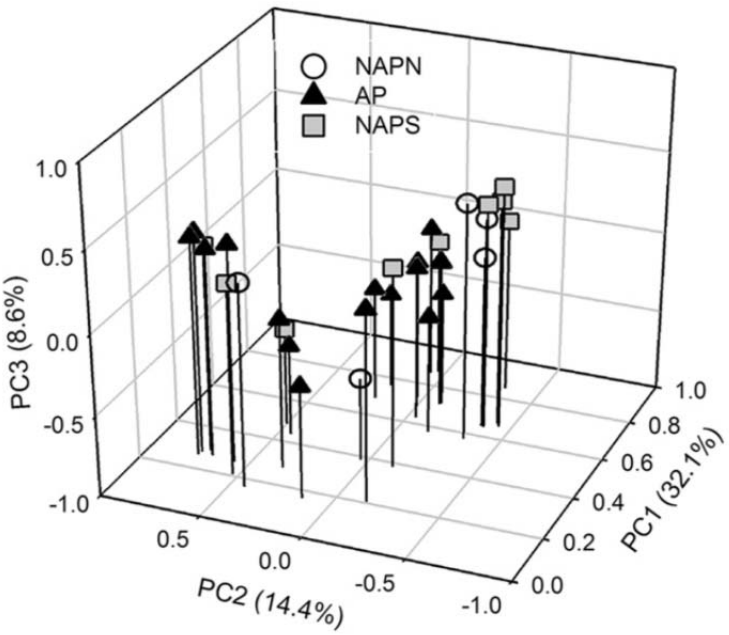

Fig. 3. Principal component analysis (PCA) comparing t-RFLP profiles of the samples located on the apatite deposit (AP) and in the flanking sites (NAPN and NAPS).

The analyses of the 16S rRNA gene sequences generated 74 non-chimeric phylotypes from the 3 AP clone libraries and 117 from the 2 NAP clone libraries. Overall, the phylotypes were distributed in 10 taxonomic groups (Fig. 4). Generally, 4 taxonomic groups (*Acidobacteria*, *Chloroflexi*, *Actinobacteria* and *Rhizobiales*) were dominant (>10% of relative abundance) in the NAP clone libraries. In the AP clone libraries, the most abundant taxonomic groups belonged to *Acidobacteria*, *Chloroflexi*, *Actinobacteria*, *Rhizobiales* and Firmicutes. In particular, in the NAP clone libraries, Actinobacteria-related sequences (27.4% of the total relative abundance) were the most diverse with 32 phylotypes, followed *Chloroflexi* related group (23.9% of the total relative abundance) with 28 phylotypes and *Rhizobiales*-related group (10.3% of the relative abundance) with 12 phylotypes. In the AP clone libraries, *Chloroflexi* (16.2% of the total relative abundance) was the most recurrent group with 12 phylotypes, followed by *Rhizobiales* (14.9% of the total relative abundance) with 11 phylotypes.

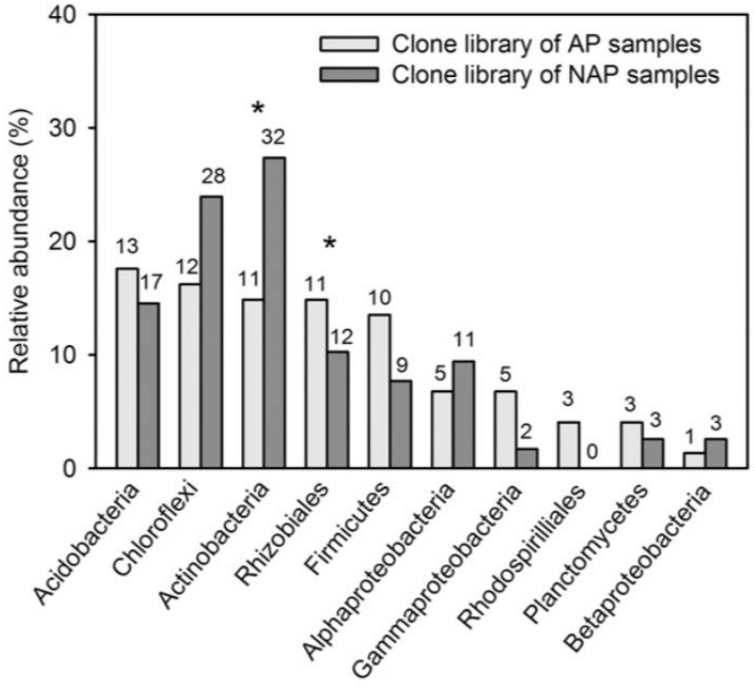

Fig. 4. Numbers of phylotypes (numbers on top of bars) and relative abundance (%) of phylotypes in clone libraries generated from samples taken on the apatite deposit (AP) and in the flanking sites (NAPN and NAPS). Asterisks indicate significant differences between the AP and NAP libraries.

Furthermore, Libshuff analysis (Fig. 5) revealed significant dissimilarities in the bacterial communities by estimating the distance between the homologous coverage curve of the NAP matrix and the heterologous coverage curve of the AP-NAP matrix. The ΔC ($(CNAP - CAP/NAP)^2$) values indicated that the main dissimilarities between the AP and NAP clone libraries were between 0.03 and 0.13 of the evolutionary distance scale. The comparison of the NAP with the AP matrix had a strong significant p-value of 0.001, whereas the inverse comparison the AP with the NAP matrix had a p-value of 0.06. To identify the taxa where the clones were significantly different, Libshuff was run on the sequences within the dominant taxonomic groups (see Table S2). These analyses revealed that *Actinobacteria* and *Rhizobiales* were significantly different ($p < 0.05$) for both the AP-NAP matrix comparisons. From the clone sequences, we performed an in-silico digestion to identify possible restriction fragments related to *Rhizobiales*, and to compare with the t-RFLP profiles of the environmental samples. Interestingly, potential restriction fragments linked to the *Rhizobiales* group appeared to have a higher relative abundance in the t-RFLP profiles of the AP samples (9.8%) than in those from the NAP samples (4.1%).

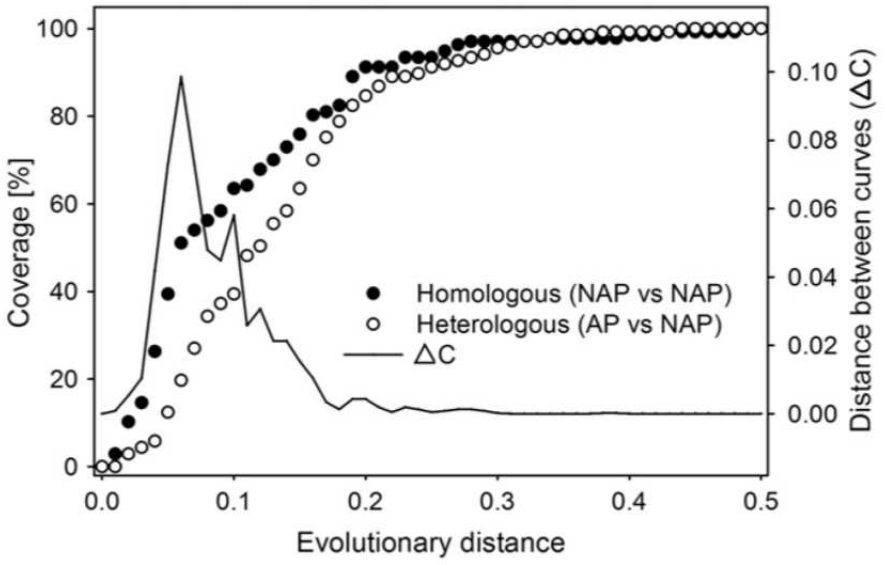

Fig. 5. Library comparison with Libshuff for 16S rRNA gene sequences ($p = 0.001$). ΔC values show the differences in evolutionary distance between the two curves with an increase of D in increments of 0.01.

4. Discussion

The aim of this study was to investigate if the composition of microbial communities on a natural apatite deposit could be distinguished from that in adjacent areas which are characterised by a different mineralogy. Our starting hypothesis was supported by previous studies demonstrating fungal and bacterial communities associated with apatite and secondary phosphate minerals in laboratory-based studies (Rogers and Bennett, 2004; Taunton et al., 2000). Moreover, microcosm laboratory-based experiments showed that the addition of rock phosphate causes a shift in microbial community structures (Carson et al., 2007). In addition, several laboratory-based studies demonstrated the ability of certain bacterial strains to survive with rock phosphate as the sole P source (Srivastava et al., 2007; Sulbarán et al., 2008). For example, through culture screening assays and experiments, representatives of bacterial genera *Rhizobium, Pseudomonas* and *Bacillus* (Kämpfer, 2007) were identified as important phosphate solubilisers.

This work is one of a few investigations attempting at addressing the association between microorganisms and soil geochemistry in nature. For example, the effect of mineralogy on microbial community structures has been previously shown along spatial gradients in relation to different mineralogical substrates (Boyd et al., 2007; Hutchens et al., 2010). Only very few studies managed to link geogenic and geochemical factors to microbial communities in soils (Reith et al., 2012). However, to our knowledge, no other study has investigated the effect of rock phosphate on microbial communities in nature.

In our work, one of the major challenges which were encountered was to identify the physical limit between the pure apatite area and the adjacent sites (non-apatite references). Our visual identification of the apatite deposits was confirmed by the geological characterisation of the sites, which permitted to identify fluorapatite as a dominant mineral of the AP region. We could therefore merge with confidence the results from the samples from the AP, NAPN, and NAPS regions of the different transects. This approach permitted to strengthen our overview of trends and patterns occurring along the apatite deposit and at its flanking sites.

Generally, the physico-chemical analyses indicated possible limiting conditions occurring at the apatite deposit in comparison to the surroundings. For example, the AP samples appeared poorer in TC and TN, a condition which may probably be related to the different vegetation type and cover of the sites (Chu and Grogan, 2010). The presence of bryophytes which was observed in most of the AP sampling spots may be an indicator of a good P bioavailability, as observed by Gordon et al. (2000). On the other hand, in most of our AP and NAP sampling spots, not only microorganisms but

also higher plants were found, and a competition for the bioavailable P is likely. Generally, Van der Heijden et al. (2008) reported on different kinds of plant-microbial interactions in oligotrophic environments. In alpine glacier forefields, for example, plants had an influence on microbial community growth and structures (Edwards et al., 2006). In early succession sites, plants may be associated with mycorrhiza for the acquisition of P (Lambers et al., 2008). In soil successional gradients, resource partitioning between microbial communities and plants has been reported (Reynolds et al., 2003). In arctic tundra soils, competition between plants and microorganisms for N and P has also been shown (Jonasson et al., 1999).

PCA analysis of the t-RFLP profiles showed that the communities developing on the apatite deposit (AP samples) were not strikingly distinct from those taken from the neighbouring areas (NAP samples). Only a few t-RFs appeared significantly more dominant in the AP samples than in the NAP samples. It was in particular from the analysis of our clone libraries, that we could deduce that the bacterial communities of the AP samples were a subpopulation of the bacterial communities found at the flanking sites. Sequence analysis indicated in fact that the AP and NAP samples shared common deep phylogenetic groups, but differed by specific minor groups. Our genetic profiling also identified half of the taxonomic groups (belonging to *Actinobacteria* and *Rhizobiales*) as potential phosphate solubilising bacteria. *Actinobacteria* have been shown to dominate the early stages of colonization of apatite–nephelite sands (Pereverzev et al., 2010), and rock-phosphate solubilising *Actinobacteria* have been isolated by Hamdali et al. (2008) in phosphate mines and by Mander et al. (2012) in grassland. Such isolates include *various* species of *Streptomycetaceae*, *Micromonosporaceae*, *Microbacteriaceae*, *Micrococcaceae* and Nocardiaceae. In our study, we also identified some sequences related to *Actinomycetales*, order of *Streptomyces* and *Micromonosporaceae* (e.g. accession number JQ267928, see Table S1). Although the phosphate-solubilising ability of *Actinobacteria* is poorly described in literature, it has been estimated that 20% of *Actinobacteria* have the ability to enhance rock dissolution (Hamdali et al., 2008; Mander et al., 2012).

As for *Rhizobiales*, they have the advantage of surviving in oligotrophic environments through their ability to fix atmospheric N combined with phosphate-solubilising activity (Alikhani et al., 2007; Antoun et al., 1998; Chabot et al., 1996). Free-living non-symbiotic organisms have also been detected in oligotrophic environments (Saito et al., 1998). Inoculation of soils with such organisms increased P availability and significantly stimulated plant growth (Wani et al., 2007; Zarei et al., 2006). Generally, *Rhizobium* has been suggested to be one of the most powerful phosphate-solubilisers (Rodríguez and Fraga, 1999) and *Rhizobium* strains have been identified to solubilise up to 620 μg P ml^{-1} from $Ca_3(PO_4)_2$ in laboratory-based experiments (Sridevi and Mallaiah, 2007). Although in the present study we did not test experimentally the extent of chemical weathering nor of bacterial-induced weathering of apatite, we cannot exclude that both processes may have contributed to the supply of bioavailable P. In a preliminary phase of the study (data not shown), we applied the screening assay in National Botanical Research Institute medium (Mehta and Nautiyal, 2000) to our soil samples and confirmed the presence of phosphate solubilising bacterial strains.

Aside from water- and organic acid-extractable P pools, and from the slight differences in community composition we observed between the AP and NAP samples, the other parameters we measured in our study did not show any clear pattern related to the presence of apatite. For example, it has been demonstrated that the main characteristic of phosphate-solubilising microorganisms under limiting conditions is to actively secrete organic acids in order to reduce soil pH and enhance apatite dissolution rate (Welch et al., 2002). However, in our study, pH was not significantly lower in the AP samples. Moreover, citrate, an important organic acid involved in phosphate solubilisation (Bolan et al., 1994; Ryan et al., 2001), was the most abundant in all samples. Weaker monocarboxylic acids such as lactate and acetate were detectable mostly in the AP samples. Such acids have been detected in liquid cultures of several phosphate-solubilising bacteria (Vazquez et al., 2000). Further analyses would be needed to assess the origin (plant derived or microbially-derived) of the measured organic acids in the different samples.

In summary, despite that we demonstrated the presence of several phosphate-solubilising groups in our samples, the effect of the natural apatite deposit on the local microbial communities was not as clear-cut as expected. While laboratory-based incubations of microbial cultures or of environmental samples permit to modulate and assess single environmental factors, *in-loco*

observations of natural ecosystems must take into account the interplay of a great variety of environmental factors which may affect the structures and activities of microbial communities.

The complex interaction of different environmental factors may lead to different response patterns, as it has been shown in recent ecological modelling studies (Lintz et al., 2011).

We measured dependent variables such as water- and H2A-1- extractable P, TC and TN and independent variables such as the rock composition. Such complexity in natural ecosystems has been studied in linear mixed models (Langenheder et al., 2010; Strickland et al., 2009). Since all these factors are interdependent, it remains open if they have a direct or indirect effect on the microbial communities. For example, in laboratory-based studies, apatite amendments in marine mesocosms (Kan et al., 2011) and in soil incubation pots (Wallander, 2000) significantly influenced microbial communities and P release from apatite. On the contrary, field-based litter bags experiments with the addition of apatite did not show significant changes in ectomycorrhizal community structures (Berner et al., 2012). In our field study system, which differs from the reported works in being a natural oligotrophic environment and subjected to the characteristic alpine climatic variations, the sole availability of an inorganic P source may not have been the strongest environmental factor controlling the composition of local soil bacterial communities.

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